

PCT/US98/17949

REC'D 08 OCT 1998

WIPO PCT

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office

September 25, 1998

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE
RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF
THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT
MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER
35 USC 111.

APPLICATION NUMBER: 60/057,067

FILING DATE: August 29, 1997

PRIORITY DOCUMENT



By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS


MARGARET BASSFORD
Certifying Officer



PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53(b)(2).

Docket Number		760100.430P1	Type a plus sign (+) inside this box →	+
INVENTOR(s)/APPLICANT(s)				
LAST NAME	FIRST NAME	MIDDLE INITIAL	RESIDENCE (CITY AND EITHER STATE OR FOREIGN COUNTRY)	
Larocca	David		Eacinitas, California	
TITLE OF THE INVENTION (280 characters max)				
METHODS USING PHAGE DISPLAY FOR SELECTING INTERNALIZING LIGANDS FOR GENE DELIVERY				
CORRESPONDENCE ADDRESS				
Carol Nottenburg, Ph.D. Seed and Berry LLP 6300 Columbia Center 701 Fifth Avenue Seattle, Washington 98104-7092				
ENCLOSED APPLICATION PARTS (check all that apply)				
<input checked="" type="checkbox"/>	Specification	Number of Pages	22	<input type="checkbox"/> Small Entity Statement
<input type="checkbox"/>	Drawing(s)	Number of Sheets		<input type="checkbox"/> Other (specify) _____
METHOD OF PAYMENT (check one)				
<input checked="" type="checkbox"/>	A check or money order is enclosed to cover the Provisional filing fees			PROVISIONAL FILING FEE AMOUNT (\$)
<input checked="" type="checkbox"/>	The Commissioner is hereby authorized to charge additional fees and credit Deposit Account Number: 19-1090			
PROVISIONAL FILING FEE AMOUNT (\$)				
\$150				

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No.☐ Yes, the name of the U.S. Government agency and the Government contract number are: _____

Respectfully submitted,

SIGNATURE

Date

29 August 1997

TYPED or PRINTED NAME

Carol Nottenburg, Ph.D.

REGISTRATION NO.

39,317

(If appropriate)



Additional inventors are being named on separately numbered sheets attached hereto.

c:\winword\cnpri\p0761

METHODS USING PHAGE DISPLAY FOR SELECTING INTERNALIZING
LIGANDS FOR GENE DELIVERY

TECHNICAL FIELD

- 5 This invention relates generally to phage display, and in particular, to selection of bacteriophages expressing peptides and proteins that bind to a cell surface receptor and internalize.

BACKGROUND OF THE INVENTION

- 10 Bacteriophage expressing a peptide on its surface has been used to identify protein binding domains, including antigenic determinants, antibodies that are specifically reactive, mutants with high affinity binding, identify novel ligands, substrate sites for enzymes. In its most common form, a peptide is expressed as a fusion protein with a capsid protein of a filamentous phage. This results in the display of the foreign protein on the surface of the phage particle. Libraries of phages are
15 generated that express a multitude of foreign proteins. These libraries are bound to a substrate or cell that presents the binding partner of interest. This screening process is essentially an affinity purification. Bound phage are recovered, propagated, and the gene encoding the foreign protein may be isolated and characterized. This technology is commonly referred to as "phage display."

- 20 Through a process called "biopanning," the specific phage carrying a peptide or protein that interacts with a protein or other moiety on a solid phase can be identified and isolated. However, in some applications, binding or binding affinity is not the sole critical parameter. For example, in gene therapy, a gene sequence needs to be introduced into a cell. In preferred methods, the gene sequence is targeted to
25 particular cells by way of a ligand / cell surface receptor interaction. Thus, the ligand must not only bind to the cells but also be internalized. A native ligand that is internalized, when used in a system for gene therapy may not be efficiently internalized. For example, both FGF2 and EGF are internalizing ligands; however, only FGF is efficient as a gene targeting ligand.

- 30 Phage libraries can be screened for internalizing ligands by biopanning on live cells and rescuing internalized phage from the cells after stripping off externally bound phage (e.g. acid elution). This method may result in recovery of undesired phage that bind very tightly or are only partially internalized.

Thus, current screening methods are inadequate for selecting peptide or protein ligands that bind to a cell surface receptor and internalize. The present invention discloses a phage display method that selects peptide or protein ligands that internalize, and further provides other related advantages.

5 SUMMARY OF THE INVENTION

Within one aspect of the present invention, a method of identifying in a library of bacteriophages expressing heterologous peptides or proteins a bacteriophage that binds to a cell surface receptor and internalizes is presented, comprising: (a) contacting a library of bacteriophages expressing a plurality of peptides with a cell, wherein the bacteriophage genome carries a gene encoding a detectable product; and (b) detecting the product; thereby identifying a bacteriophage expressing a heterologous peptide that binds to a cell surface receptor and internalizes.

In another aspect, the invention provides a method of isolating cells that have internalized a bacteriophage present in a library of bacteriophages expressing heterologous peptides or proteins, comprising: (a) contacting a library of bacteriophages expressing a plurality of peptides with a cell, wherein the bacteriophage genome carries a gene encoding a detectable product; (b) detecting the product; and (c) isolating cells that express the product.

In yet another aspect, the invention provides a method of selecting a bacteriophage expressing a heterologous peptide that binds to a cell surface receptor and internalizes, comprising: (a) contacting a library of bacteriophages expressing a plurality of peptides with a cell, wherein the bacteriophage genome carries a gene encoding a detectable product; (b) detecting the product; and (c) recovering the bacteriophage gene encoding the peptide from cells expressing the product; thereby selecting a bacteriophage expressing a heterologous peptide that binds to a cell surface receptor and internalizes.

In yet another aspect, a method is provided for selecting a bacteriophage expressing a heterologous peptide that binds to a cell surface receptor and internalizes, comprising: (a) contacting a library of bacteriophages expressing a plurality of peptides with cells, wherein the bacteriophage genome carries a gene encoding a selectable product; (b) incubating the cells under selective conditions; and (c) recovering the bacteriophage gene encoding the peptide from the selected cells; thereby selecting a bacteriophage expressing a heterologous peptide that binds to a cell surface receptor and internalizes.

In preferred embodiments, the library is a cDNA library, an antibody gene library, or a random peptide gene library. In other preferred embodiments, the detectable product is selected from the group consisting of green fluorescent protein, β -galactosidase, membrane bound protein, secreted alkaline phosphatase, chloramphenicol acetyltransferase, luciferase, human growth hormone and neomycin phosphotransferase.

In other embodiments, the cell surface receptor is FGF-R or erbB2. In yet other embodiments, the cells are tumor cells or endothelial cells. The cells may be isolated by flow cytometry, for example.

The bacteriophage are filamentous phage or lambdoid phage in preferred embodiments.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are set forth below which describe in more detail certain procedures or compositions (e.g., plasmids, etc.), and are therefore incorporated by reference in their entirety.

DETAILED DESCRIPTION OF THE INVENTION

As noted above, the present invention provides a method of phage display that identifies and/or selects for peptide and protein ligands that bind and internalize on the basis of expression of a transgene that is carried on the phage genome.

Briefly, in the present invention, a library of antibodies, cDNAs, or genes encoding random peptides is cloned into a coat protein (e.g., gene III protein of filamentous phage) of a bacteriophage. The phage genome also contains an "expression cassette" encoding a transgene placed downstream from a cell promoter that is active in the cells to be infected. The transgene may be a selectable gene product and/or a detectable marker. Phage are contacted with test cells and expression of the transgene is monitored or selected. Phage that internalize will confer the phenotype of the transgene, such as drug resistance or expression of a fluorescing protein. The cells may be isolated on the basis of transgene expression. For example, when the transgene is a drug resistance gene, cells are grown in the presence of the drug, such that only those cells receiving and expressing the transgene are propagated. The gene(s) that are fused with the coat protein and that promoted cell binding and internalization are recovered from the selected cells by a suitable method.

35

1. PHAGE DISPLAY VECTORS AND METHODS

A variety of bacteriophages may be used within the context of the present invention. Such phage include the filamentous phages, lambda, T4, MS2, and the like. A preferred phage is a filamentous phage, such as M13 or Φ .

- 5 Phage that present the foreign protein or peptide as a fusion with a phage coat protein are engineered to contain the appropriate coding regions. A variety of bacteriophage and coat proteins may be used. Examples include, without limitation, M13 gene III, gene VIII; fd minor coat protein pIII (Saggio et al., *Gene* 152: 35, 1995); lambda D protein (Sternberg and Hoess, *Proc. Natl. Acad. Sci. USA* 92: 1609, 1995; Mikawa et al., *J. Mol. Biol.* 262: 21, 1996); lambda phage tail protein pV (Maruyama et al., *Proc. Natl. Acad. Sci. USA* 91: 8273, 1994; U.S. Patent No. 5627024); f1 coat protein (WO 96/11947; DD 292928; DD 286817; DD 300652); ϕ 29 tail protein gp9 (Lee, *Virology* 69: 5018, 1995); MS2 coat protein; T4 small outer capsid protein (Ren et al., *Protein Sci.* 5: 1833, 1996), T4 nonessential capsid scaffold
- 15 protein IP1II (Hong and Black *Virology* 194: 481, 1993), or T4 lengthened fibrin protein gene (Efimov, *Virus Genes* 10: 173, 1995); PRD-1 gene III; Q β 3 capsid protein (as long as dimerization is not interfered with); and P22 tailspike protein (Carbonell and Villaverde, *Gene* 176: 225, 1996). Techniques for inserting foreign coding sequence into a phage gene are well known (see e.g., Sambrook et al.,
- 20 *Molecular Cloning: A Laboratory Approach*, Cold Spring Harbor Press, NY, 1989; Ausubel et al., *Current Protocols in Molecular Biology*, Greene Publishing Co., NY, 1995).

In the preferred filamentous phage system, a wide range of vectors are available (see, Kay et al., *Phage Display of Peptides and Proteins: A Laboratory*

- 25 *Manual*, Academic Press, San Diego, 1996). The most common vectors accept inserts in gene III or gene VIII. Furthermore, the foreign gene can be inserted directly into the phage genome or into a phagemid vector. Methods of propagation of filamentous phage and phagemids are well known.
- Filamentous phage vectors generally fall into two categories: phage
- 30 genome and phagemids. Either type of vector may be used within the context of the present invention. Many such commercial vectors are available. For example, the pEGFP vector series (Clontech; Palo Alto, CA), M13mp vectors (Pharmacia Biotech, Sweden), pCANTAB 5E (Pharmacia Biotech), pBluescript series (Stratagene Cloning Systems, La Jolla, CA) and others may be used. One particularly useful commercial
- 35 phagemid vector is pEGFP-N1, which contains a green fluorescent protein (GFP) gene under control of the CMV immediate-early promoter. This plasmid also

includes an SV40 origin of replication to enhance gene expression by allowing replication of the phagemid to high copy number in cells that make SV40 T antigen.

Other vectors are available in the scientific community (see e.g., Smith, in *Vectors: A Survey of Molecular Cloning Vectors and their Uses*, Rodriguez and Denhardt, eds., Butterworth, Boston, pp 61-84, 1988) or may be constructed using standard methods (Sambrook et al., *Molecular Biology: A Laboratory Approach*, Cold Spring Harbor, NY, 1989, Ausubel et al., *Current Protocols in Molecular Biology*, Greene Publishing, NY, 1994) guided by the principles discussed below.

The source of the ligand (e.g., gene, gene fragment or peptide encoding sequence) may be for example, derived from a cDNA library, antibody library or random peptide library. Alternatively, the ligand may be from a library of random mutations of a known ligand.

When a cDNA library is used, the starting cDNA is synthesized from mRNA isolated from the source tissue or cell line from which the desired ligand originates. cDNA is then amplified using primers containing sequences of appropriate restriction enzyme sites for insertion into the desired vector. Alternatively, commercially available cDNA libraries (e.g. Clontech; Palo Alto, CA) may be amplified for insertion into the vector.

Similarly, libraries of antibody fragments can be made from mRNA isolated from the spleen cells of immunized animals (immunized for example with whole target cells or membranes) or subcloned from existing antibody libraries from immunized or naive animals. Random peptides are subcloned from libraries that are commercially available (New England Biolabs; MA) or can be synthesized and cloned using previously described methods (in Kay et al, *supra*).

Phage display libraries of random mutations of known ligands for improved gene delivery are performed in the same manner as described for screening random peptide libraries. Random mutations of the native ligand gene may be generated using DNA shuffling as described by Stemmer (Stemmer, P., *Nature*, 370, 389-391). Briefly, in this method, the ligand is amplified and randomly digested with DNase I. The 50-300 base pair fragments are reassembled in an amplification performed without primers and using *Taq* DNA polymerase or similar enzyme. The high error rate of this polymerase introduces random mutations in the fragments that are reassembled at random thus introducing combinatorial variations of different mutations distributed over the length of the gene. Error prone amplification may alternatively be used to introduce random mutations (Bartell and Szostak, *Science*, 261:1411, 1993). The ligand may be mutated by cassette mutagenesis (Hutchison et

al., *In Methods in Enzymology* 202: 356-390), in which random mutations are introduced using synthetic oligonucleotides and cloned into the ligand to create a library of ligands with altered binding specificities. Additional mutation methods can be used. Some additional methods are described in Kay et al., *supra*.

- 5 If a cDNA library cannot be generated because, for example, the source of the desired ligand is not available or is unknown, random peptide libraries or a cDNA library from placenta may be used as a starting point for screening. Methods for construction of random peptide libraries may be found, for example, in Kay et al., *supra*. Briefly, the random peptides are encoded by DNA assembled from degenerate
- 10 oligonucleotides and inserted into one of the bacteriophage vectors described herein. Several different strategies may be used to generate random peptides. For example, triplets of NNN, wherein each N is an equimolar representation of all four nucleotides, will generate all 20 amino acids (as well as 3 stop codons). Alternative strategies use NN(G/T) and NN(G/C), which results in 32 codons that encodes all 20 amino acids
- 15 and only 1 stop codon. Other strategies utilize synthesis of mixtures of trinucleotide codons representing all 20 amino acids and no stop codons. Once the oligonucleotides are synthesized, they are assembled as double strands by a variety of schemes, one of which involves synthesis of the complementary strand (see Kay et al., *supra*).

- 20 In addition to the ligand/coat protein fusion, the vector contains a gene whose product can be detected or selected for. As referred to herein, a "reporter" gene is one whose product can be detected, such as by fluorescence, enzyme activity on a chromogenic or fluorescent substrate, and the like or selected for by growth conditions. Such reporter genes include, without limitation, green fluorescent protein
- 25 (GFP), β -galactosidase, chloramphenicol acetyltransferase (CAT), luciferase, neomycin phosphotransferase, secreted alkaline phosphatase (SEAP), and human growth hormone (HGH). Selectable markers include drug resistances, such as neomycin (G418), hygromycin, and the like.

- The marker gene is in operative linkage with a promoter. Any
- 30 promoter that is active in the cells to be transfected can be used. The vector should also have a viral origin of replication and a packaging signal for assembling the vector DNA with the capsid proteins.

- Most applications of the present invention will involve transfection of mammalian cells, including human, canine, feline, equine, and the like. The choice of
- 35 the promoter will depend in part upon the targeted cell type. Promoters that are suitable within the context of the present invention include, without limitation,

constitutive, inducible, tissue specific, cell type specific, temporal specific, or event-specific, although constitutive promoters are preferred.

Examples of constitutive or nonspecific promoters include the SV40 early promoter (U.S. Patent No. 5,118,627), the SV40 late promoter (U.S. Patent No. 5,118,627), CMV early gene promoter (U.S. Patent No. 5,168,062), bovine papilloma virus promoter, and adenovirus promoter. In addition to viral promoters, cellular promoters are also amenable within the context of this invention. In particular, cellular promoters for the so-called housekeeping genes are useful (e.g., β -actin). Viral promoters are generally stronger promoters than cellular promoters.

In preferred embodiments, the phage has an origin of replication suitable for the transfected cells. Viral replication systems, such as EBV ori and EBNA gene, SV 40 ori and T antigen, or BPV ori, may be used. Other mammalian replication systems may be interchanged. As well, the replication genes may cause high copy number. Expression of therapeutic genes from the phage genome may be enhanced by increasing the copy number of the phage genome. In one method, the SV40 origin of replication is used in the presence of SV40 T antigen to cause several hundred thousand copy number. The T antigen gene may be already present in the cells, introduced separately, or included in the phage genome under the transcriptional control of a suitable cell promoter. Other viral replication systems for increasing copy number can also be used, such as EBV origin and EBNA.

In other embodiments, peptides or other moieties that allow or promote the escape of the vectors (and any molecule attached thereto or enclosed therein) from the endosome are incorporated and expressed on the surface of the bacteriophage. Such "other moieties" include molecules that are not themselves peptides but which have the ability to disrupt the endosomal membrane, thereby facilitating the escape of the vector, and molecules that otherwise mimic the endosomal escape properties of the within-described peptide sequences (see, e.g., published International App. No. WO96/10038, the disclosures of which are incorporated by reference herein).

Peptide sequences that confer the ability to escape the endosome are particularly preferred. Such sequences are well known and can be readily fused covalently or genetically to a coat protein, such as gene III or gene VIII of filamentous phage. Although fusion of one or more peptide sequences to a coat protein is described herein as a preferred embodiment, it should be understood that other methods of attachment -- and other moieties besides peptides -- are useful as disclosed herein.

Thus, an example of a dual display filamentous phage presents a ligand (e.g., FGF) as a fusion to gene III and an endosomal escape peptide fused to gene VIII. The locations of the ligand and escape sequences are interchangeable. Escape sequences that are suitable include, without limitation, the following exemplary sequences: a peptide of *Pseudomonas* exotoxin (Donnelly, J.J., et al., *PNAS*, 90, 3530-3534, 1993); influenza peptides such as the HA peptide and peptides derived therefrom, such as peptide FP13; Sendai Virus fusogenic peptide; the fusogenic sequence from HIV gp1 protein; Paradoxin fusogenic peptide; and Melittin fusogenic peptide (see WO 96/41606).

Another sequence that may be included in a vector is a sequence that facilitates trafficking proteins into the nucleus. These so-called nuclear translocation or nuclear localization sequences (NLS) are generally rich in positively charged amino acids. Because the carboxyl terminus of gene VIII protein of filamentous phage already carries a positive charge, increased charge and likelihood of nuclear transport may be enhanced by fusing known mammalian cell NLS sequences to the gene VIII protein. NLS fusions to other coat proteins of filamentous phage may be substituted.

Examples of NLS sequences include those resembling the short basic NLS of the SV40 T antigen; the bipartite NLS of nucleoplasmin; the ribonucleoprotein sequence A1; the small nuclear ribonucleoprotein sequence U1A, and human T-lymphocyte virus-1 Tax protein. Other useful NLS sequences include the HIV matrix protein NLS; and the nuclear translocation components importin/hSRP1 and Ran/TC4; the consensus sequence KXX(K/R) flanked by Pro or Ala; the nuclear translocation sequence of nucleoplasmin; or the NLS from antennapedia. (See WO 96/41606)

As described herein, the library is then propagated in the display phage by transfection of a suitable bacteria host (e.g., DH5 α F' for filamentous phages), and growing the culture, with the addition of a replication-competent helper virus if necessary, overnight at 37°C. The phage particles are isolated from the culture medium using standard protocols.

Infection of mammalian cells with phage is performed under conditions that block entry of wild type phage into cells (Barry et al, *Nature Med.* 2:299-305, 1996). Phage are added directly to cells at titers of $<10^8$ CFU/ml in a buffer, such as PBS with 0.1% BSA or other suitable blocking agents, and allowed to incubate with the cells at 37°C or on ice. The amount of phage added to cells will depend in part upon the complexity of the library. For example, a phage display library containing 10^6 members has each member represented 10^3 times in 1 ml of a

typical phage titer of 10^{12} colony forming units/ml, and, 10^{15} phage would be needed to represent 10^6 copies of each member of a peptide library with 10^9 members. Generally cells are infected at an MOI of about 10^4 phage: cell.

5 II. DETECTION/SELECTION OF TRANSGENE EXPRESSION

The phage display library is ultimately screened against the target tissue or cell line. Screening can be performed *in vitro* or *in vivo*. The criteria for a positive "hit" is that the phage must be able to bind, be internalized, translocate to the nucleus, uncoat and replicate and express the genomic DNA containing the reporter gene in the target cell. Thus, only phage that express a reporter gene are selected.

The test cells may be any cells that express a receptor of choice or are a cell type or source for which gene therapy is destined. Thus, in some instances, the receptor may be unknown. In such cases, the selection method can be used to isolate a ligand for an receptor without a known ligand (orphan receptor) such as erbB3. Briefly, the orphan receptor is cloned into a mammalian expression vector that also contains a selectable drug resistance gene and transfected into mammalian cells, such as COS cells. Stable transfectants that overproduce the orphan receptor are selected by cultivation in the appropriate drug. This receptor-transformed COS cell line is then used as the cell line for selection of ligand-displaying phage.

Tissue-specific or tumor-specific ligands can be selected by pre-absorption of the phage library against normal or non-targeted tissues of cell cultures. The selection process can also be applied *in vivo* by injecting the library into tumor-bearing mice. The tumor is removed from the mouse 48-72h after injection. A cell suspension is prepared and phage genome bearing cells selected by one of the methods described herein. The gene whose product allows entry and expression of the phage genome is then isolated from the drug resistant cell colonies.

Screening may be performed directly against the target cells with no pre-screening or pre-enrichment. Pre-screening or pre-enrichment may be used and can be especially helpful when either too few or too many hits are observed. Enrichment for cell binding may improve detectability if no hits are found in the initial screen. A prescreen to remove phage that bind non-specific cells surface proteins may reduce non-specific hits if there are too many initial hits. Infection of at least 10^4 target cells is performed with at least 10^{12} phage. The cells are incubated for at least 2 hours in PBS/BSA and washed extensively (Barry et al., *Nature Med.* 2, 299-305, 1996). The cells are incubated in media at 37°C for 48-96 hours and then detected or selected on the basis of expression of the reporter gene.

Assays for each of these reporter gene products are well known. For example, GFP is detected by fluorescence microscopy or flow cytometry, SEAP is detected in medium using a fluorescent substrate (Clontech; Palo Alto, CA), human growth hormone may be detected in medium by a simple and sensitive radioimmune assay (Nichols Institute; CA). Western blotting and ELISA may also be used to immunologically detect and measure the presence of reporter gene product. Alternatively, the message for the reporter gene is detected using RNase probe protection or fluorescent probe hybridization. For isolation of the phage vector DNA and insert, any technique that can identify and isolate the cells expressing detectable marker product may be used. Flow cytometry, in particular, is well suited for detecting fluorescence in or on a cell and isolating that cell.

When the reporter gene is a selectable marker, the cells are grown in selective conditions. Depending upon the marker, the conditions may be a particular growth temperature, addition of a drug, or the like. In the examples provided herein, the selectable marker is neomycin transferase, which confers G418 resistance on mammalian cells. Briefly, the cells are grown in the presence of G418 for 7-14 days or until resistant colonies are visible microscopically. Colonies are picked, and phage vector DNA recovered, conveniently as amplification of the insert.

Alternatively, multiple rounds of infection and selection are performed to reduce the complexity of the infecting phages. For example, drug-resistant colonies are pooled and the selected inserts amplified and cloned back into the phage display vector for a new round of infection. When the reporter is fluorescent, flow cytometry can be used to select the strongest fluorescing cells to select the most highly efficient gene delivery ligands. More stringent screening conditions also include higher selective drug concentrations. At the completion of a selection process, representative phage clones may be subjected to DNA sequence analysis to further characterize gene delivery ligands.

Screening *in vivo* may be performed similar to methods for targeting organs or xenograft tumors using phage displayed peptides (Pasqualini et al., *Nature Biotech.* 15: 542-546, 1997; Pasqualini et al., *Nature* 380: 364-366, 1996), except that the organs or tumors are examined for reporter gene expression instead of the presence of phage. Briefly, a phage display library is injected intravenously into animals, generally mice, and organs or tumor samples are tested for reporter gene function at 48-96 hours after injection. Tumor cells may be cultured in selective conditions or sorted by flow cytometry or other method to enrich for cells that express the phage transducing gene. The ligand encoding sequences can be amplified from

selected cells as described above. As in *in vitro* screening, repeated rounds of infection and rescreening, alone or in combination with increased screening stringency, may be used to obtain the most efficient gene delivery ligands.

Specificity may also be examined *in vitro* using a panel of non-targeted
 5 and targeted cell lines and detecting expression of the phage transducing gene. Competition studies with free ligand or a neutralizing antibody to the ligand or receptor are used to confirm specific entry of phage via the ligand receptor complex. Alternatively, the cloned receptor for the ligand can be overexpressed in a cell line that normally does not express that receptor. Phage internalization and expression
 10 into the stable transfectants expressing the receptor but not the parent cell line indicates the specificity of the ligand for its receptor on receptor bearing cells.

Ligands that are identified as gene targeting ligands using the selection strategies described herein may be further tested for specificity by reporter gene expression in target and non-target cells and tissues. The ligand may also be tested in
 15 a variety of gene delivery methods, such as ligand-polylysine/DNA complexes (Sosnowski et al., *J. Biol. Chem.* 272: 33647-33653, 1996) or retargeted adenovirus gene delivery (Goldman et al., *Cancer Research* 57:1447-1451, 1997).

The specificity of the targeting ligand may alternatively be determined *in vivo* by biodistribution analysis using one of the reporter genes described herein,
 20 such as luciferase. At various time points, mice injected with the ligand displaying phage are sacrificed and tissues examined for the presence of phage in non-targeted tissues by immunohistochemistry, an enzymatic assay that detects reporter product activity, or the like.

25 III. USES

The methods described herein are designed to select cDNAs, Fabs, SFV, random peptides, and the like for discovery of new ligands. It can also be used to select mutated and gene-shuffled versions of known ligands for targeting ability.

These ligands may have increased transduction efficiency (as measured
 30 by an increase in the percentage of infected cells that express the reporter gene); increased expression of the reporter gene (as measured by intensity of reporter gene expression) in the phage transduced cells; increased specificity of transduction for target cells (as measured for ligand specificity); increased stability of the ligand (as measured by ability to target the ligand *in vivo* to tumor cells); increased affinity for
 35 receptor (e.g., removing dimerization requirements for ligands that dimerize); elimination of the need for cofactors (e.g., development of an FGF variant that binds

with high affinity to the FGF receptor but not to heparin); altered specificity for receptor subtypes (e.g., an FGF variant that reacts with only one of the four FGF receptors).

The ligands identified by the methods described herein may be used as targeting agents for delivering therapeutic agents to cells or tissues. For example, a therapeutic gene can be incorporated into the phage genome and delivered to cells via phage bearing the gene delivery ligand on its protein coat.

As used herein, a "therapeutic nucleic acid" or "therapeutic gene" describes any nucleic acid molecule used in the context of the invention that effects a treatment, generally by modifying gene transcription or translation. It includes, but is not limited to, the following types of nucleic acids: nucleic acids encoding a protein, ribozyme, antisense nucleic acid, DNA intended to form triplex molecules, protein binding nucleic acids, and small nucleotide molecules. As such, the product of the therapeutic gene may be DNA or RNA. These genes sequences may be naturally-derived sequences or recombinantly derived. A therapeutic nucleic acid may be used to effect genetic therapy by serving as a replacement for a defective gene, by encoding a therapeutic product, such as TNF, or by encoding a cytotoxic molecule, especially an enzyme, such as saporin. The therapeutic nucleic acid may encode all or a portion of a gene, and may function by recombining with DNA already present in a cell, thereby replacing a defective portion of a gene. It may also encode a portion of a protein and exert its effect by virtue of co-suppression of a gene product.

As discussed above, the therapeutic gene is provided in operative linkage with a selected promoter, and optionally in operative linkage with other elements that participate in transcription, translation, localization, stability and the like.

The therapeutic nucleotide composition of the present invention is from about 20 base pairs to about 100,000 base pairs in length. Preferably the nucleic acid molecule is from about 50 base pairs to about 50,000 base pairs in length. More preferably the nucleic acid molecule is from about 50 base pairs to about 10,000 base pairs in length. Even more preferably, it is a nucleic acid molecule from about 50 pairs to about 4,000 base pairs in length.

The bacteriophages provided herein are useful in the treatment and prevention of various diseases, syndromes, and hyperproliferative disorders, such as restenosis, other smooth muscle cell diseases, tumors, such as melanomas, ovarian cancers, neuroblastomas, pterygia, secondary lens clouding, and the like. As used herein, "treatment" means any manner in which the symptoms of a condition, disorder

or disease are ameliorated or otherwise beneficially altered. Treatment also encompasses any pharmaceutical use of the compositions herein. As used herein, "amelioration" of the symptoms of a particular disorder refers to any lessening, whether permanent or temporary, lasting or transient, that can be attributed to or
5 associated with administration of the composition.

In certain embodiments, the compositions of the present invention may be used to treat angiogenesis-dependent diseases. In these diseases, vascular growth is excessive or allows unwanted growth of other tissues by providing blood supply. These diseases include angiofibroma, arteriovenous malformations, arthritis,
10 atherosclerotic plaques, corneal graft neovascularization, delayed wound healing, diabetic retinopathy, granulations due to burns, hemangiomas, hemophilic joints, hypertrophic scars, neovascular glaucoma, nonunion fractures, Osler-weber syndrome, psoriasis, pyogenic granuloma, retrolental fibroplasia, scleroderma, solid tumors, trachoma, and vascular adhesions.

By inhibiting vessel formation (angiogenesis), unwanted growth may be slowed or halted, thus ameliorating the disease. In a normal vessel, a single layer of endothelial cells lines the lumen, and growth of the vessel requires proliferation of endothelial cells and smooth muscle cells.
15

As well, the phages of the present invention may be used to treat tumors. In these diseases, cell growth is excessive or uncontrolled. Tumors suitable for treatment within the context of this invention include, but are not limited to, breast tumors, gliomas, melanomas, prostate cancer, hepatomas, sarcomas, lymphomas, leukemias, ovarian tumors, thymomas, nephromas, pancreatic cancer, colon cancer, head and neck cancer, stomach cancer, lung cancer, mesotheliomas, myeloma,
20 neuroblastoma, retinoblastoma, cervical cancer, uterine cancer, and squamous cell carcinoma of skin. For such treatments, ligands are chosen to bind to cell surface receptors that are generally preferentially expressed in tumors.

Through delivery of the compositions of the present invention, unwanted growth of cells may be slowed or halted, thus ameliorating the disease. The methods utilized herein specifically target and kill or halt proliferation of tumor cells having receptors for the ligand on their surfaces.
25

The phages may also be used to treat or prevent atherosclerosis and stenosis, a process and the resulting condition that occurs following angioplasty in which the arteries become reclogged. Generally, treatment of atherosclerosis involves widening a stenotic vascular lumen, permitting greater blood flow and oxygenation to the distal tissue. Unfortunately, these procedures induce a normal wound healing
30

response in the vasculature that results in restenosis. Of the three components to the normal vascular response to injury, thrombosis, elastic recoil and smooth muscle cell proliferation, anti-thrombotics/platelet inhibitors and vascular stents effectively address acute/subacute thrombosis and elastic recoil, respectively. However, no therapy can modify the vascular remodeling that is due to proliferation of smooth muscle cells at the lesion, their deposition of extracellular matrix and the subsequent formation of a neointima. Accordingly, restenosis remains a significant clinical problem.

- Wound response also occurs after other interventions, such as balloon angioplasty of coronary and peripheral vessels, with or without stenting; carotid endarterectomies; vein grafts; and synthetic grafts in peripheral arteries and arteriovenous shunts. Although the time course of the wound response is not well defined, if the response can be suppressed for a short term (approximately 2 weeks), a long term benefit is achieved.

- The following examples are offered by way of illustration, and not by way of limitation.

EXAMPLES

EXAMPLE 1

PREPARATION OF PHAGEMID DISPLAY VECTOR

5

The phagemid pEGFP-N1 (Clontech; Palo Alto, CA) contains a green fluorescent protein (GFP) gene under control of the CMV immediate-early promoter. The CMV promoter is highly active in a large variety of mammalian cell lines, however, other mammalian cell promoters can be used.

10

Phagemid pEGFP-N1 is propagated in the *E. coli* host strain, DH5 α F' to allow for super infection with the M13 helper bacteriophage, M13K07. Phagemid particles are prepared according to known methods (see for example, Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, 1989; and Rider et al. in *Phage Display of Peptides and Proteins: A Laboratory Manual*, Academic Press 1996). A fresh bacterial colony containing pEGFP-N1 is suspended in 3 ml of 2X YT medium, M13K07 is added to a final titer of 2×10^7 pfu/ml and the culture incubated for 1 hour at 37 °C. Kanamycin is added to a final concentration of 70 μ g/ml and incubation continued for 14-18 hours. The bacterial culture is pelleted at 12,000 x g for 10 minutes, and the supernate is transferred to a fresh tube. The phagemid particles are precipitated by the addition of 1/3 volume of 30% polyethylene glycol (PEG)-1.5M NaCl. The solution is vortexed and chilled for 1 hour on ice or overnight at 4°C. The phage is pelleted by centrifugation at 12,000 x g for 10 minutes at 4 °C. The pellet is thoroughly drained and resuspended in 1/10 original volume of PBS (phosphate buffered saline pH 7.4). The PEG precipitation is repeated and the pellet resuspended in 1/20 volume of PBS. The phagemid suspension is heat pasteurized at 65°C for 5 minutes, filtered through a 0.45 μ M filter, and stored at 4°C.

25

30

EXAMPLE 2

CONSTRUCTION OF FGF2-CONTAINING PHAGE DISPLAY VECTORS

In the following examples, a phage that displays FGF2 on its surface is used to bind to the FGF2 receptor on mammalian cells and be internalized. An FGF gene that contains Cys \rightarrow Ser amino acid changes at residue 96 is subcloned from the expression plasmid pET11-FGF2-3 into the phagemid display vector pCANTAB 5 E (Pharmacia Biotech; Piscataway,NJ) or M13 type 3 or 33 for gene III fusions (see

35

Kay, B.K. et al *Phage Display of Peptides and Proteins: A Laboratory Manual*, Academic Press, 1996, McConnell, S.J. et al., *Mol. Divers.* 1:165-176, 1996). Similarly, FGF2-3 is cloned into M13 type 8 or 88 vector for fusion to gene VIII protein (Roberts et al., *Methods Enzymol.* 267:68-82, 1996; Markland W. et al., *Gene* 109:13-19, 1991).

To facilitate insertion, the FGF2-3 gene is amplified using primers that contain appropriate restriction endonuclease sites into the phage vector gene III or VIII genes. The resulting phage express immunologically and biologically reactive FGF2 as detected by anti-FGF2 antibody probing of phage plaque immunoblots. Binding of the FGF2 fusion phage to FGF2 receptor is assessed by ELISA in which recombinant FGF2 receptor is attached to the solid phase and an anti-phage antibody is used as the primary detection antibody.

EXAMPLE 3

BINDING AND INTERNALIZATION OF FGF2-EXPRESSING PHAGE

The FGF2-expressing phage are also assayed for high affinity receptor binding and internalization in receptor bearing cells by immunolocalization and fluorescence microscopy (Hart, *J. Biol. Chem.* 269:12468-12474, 1994; Barry et al, *Nature Med.* 2:299-305, 1996; Li, *Nature Biotech.* 15:559-563, 1997).

Infection of mammalian cells with FGF2-expressing phage is performed under conditions that block entry of wild type M13 phage into cells except chloriquine is not used (Barry et al., *supra*). Phage are added directly to cells at titers of $<10^{10}$ CFU/ml in PBS with 0.1% BSA or other suitable blocking agents and incubated at 37°C or on ice for at least 1 hour. The cells are then washed extensively in PBS, fixed in 2% paraformaldehyde, and permeabilized in 100% methanol at room temperature for 10 minutes. Cells are incubated with rabbit anti-M13 antibody in PBS/BSA for 1 hour. The primary antibody is detected with a phycoerythrin labeled anti-rabbit antibody. Surface bound (incubated on ice) or internalized (37°C incubation) phage are detected by fluorescence microscopy.

EXAMPLE 4

CONSTRUCTION OF A REPORTER GENE AND A DRUG RESISTANCE GENE IN PHAGE DISPLAY VECTORS

A GFP expression cassette consisting of the GFP gene (Cormack et al., *Gene*, 173, 33-37, 1996) under control of a CMV promoter, a neomycin

phosphotransferase gene under control of the SV40 early gene promoter, and an SV40 origin of replication are cloned into a gene III phagemid vector such as pCANTAB 5E using standard methods (Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, 1989)). The resulting phage is designated pmaM13. The same phagemid genome also containing FGF2-3 fused to gene III is designated pFGF-maM13. Similar constructs are also made with M13 phage type 33 and gene VIII phagemid and phage vectors. Recombinant phage displaying FGF2 on the coat and carrying the mammalian expression cassettes including the SV40 replication origin are prepared by phagemid rescue with M13K07 (or suitable helper phage) are added to COS cells as described above. GFP expression is detected by fluorescence microscopy, fluorometry, and flow cytometry at 48-96 hours after phage addition. Drug resistant cells are selected with G418.

EXAMPLE 5

15 SELECTION OF FGF2-EXPRESSING PHAGE FROM A MIXED POPULATION

A M13 phage display library of random or unknown sequences is spiked with pFGF-maM13 phage. The mixture is used to infect COS cells as described above. The cells are washed extensively to remove non-specifically bound
20 phage. Cells are replated 48-96 hours later at a 1 to 10 dilution and grown in G418 to select only cells that receive the transducing phage gene. Alternatively, the GFP expressing cells are isolated by flow cytometry using an excitation wavelength of 488 and emission wavelength of 510.

DNA is extracted from G418-resistant cells and the FGF2 sequence is
25 amplified. The amplification primers have sequences complementary to phage sequences located on each side of the FGF2 sequence in the gene III coding sequence. Detection of the FGF2 sequences in selected COS cells that are infected with a mixture of phage where the pFGF-maM13 phage is diluted at least 1:10,000 with the random sequence phage library demonstrates feasibility of the technique.

30

EXAMPLE 6

SCREENING LIBRARIES FOR GENE DELIVERY LIGANDS

35 If the source of the desired ligand is not known, random peptide libraries or a cDNA library from placenta is used as a starting point for cDNA library screening. The library is amplified in the maM13-33 phage by infecting DH5 α F' (or

other suitable host) bacteria, growing the culture overnight at 37°C and isolating the phage from the culture medium using standard protocols. A cDNA library containing 10^6 members has each member represented 10^6 times in a typical phage titer of 10^{12} colony forming units/ml.

- 5 The amount of phage used to infect is adjusted to the complexity of the library. A titer of 10^{15} phage would be needed to represent 10^6 copies of each member of a peptide library with 10^9 members.

- 10 The completed maM13 phage library is screened against the target tissue or cell line. Screening can be performed in-vitro or in-vivo. The criteria for a positive "hit" is that the phage must be able to bind, be internalized, translocate to the nucleus, uncoat and replicate and express the genomic DNA containing the reporter gene in the target cell. Thus, only transduced target cells are selected either by GFP expression and cell sorting or drug resistance. Screening is performed directly against the target cells with no prescreening or enrichment. Enrichment for cell binding is performed if no hits are found in the initial screen. A prescreen to select out phage that bind non-specific cells surface proteins is performed to reduce non-specific hits or if there are too many initial hits. Infection of at least 10^6 target cells is performed with at least 10^{12} phage. The cells are incubated for at least 2 hours in PBS and washed extensively as described by Barry (Barry, M. et al, Nature Med., 2, 299-305, 1996).
- 20 The cells are incubated in media at 37°C for 48-96 hours and selected in the appropriate drug (e.g., G418) for 7-14 days or until resistant colonies are visible microscopically. Drug resistant colonies are pooled, and the selected cDNAs amplified and subcloned back into the maM13-33 phage vector using PCR and standard molecular biology methods. Alternatively individual colonies are screening.
- 25 Representative phage clones are sequenced to identify potential gene delivery ligands. Repeated rounds of infection and selection are performed to reduce the complexity of the selected clones. More stringent screening conditions such as increased selective drug concentrations or FACS sorting or the strongest fluorescent cells are performed in the later screens to select the most highly efficient gene delivery ligands from the
- 30 initial screening.

- Screening *in-vivo* is performed using methods previously described by Pasqualini for targeting organs or xenograft tumors using phage displayed peptides (Pasqualini, R. et al, Nature Biotechnology, 15, 542-546 (1997); Pasqualini, R. et al., Nature, 380, 364-366 (1996)) except that the organs or tumors are examined for
- 35 reporter gene expression instead of the presence of phage. The phage library is injected intravenously into mice and organs or tumor samples tested for reporter gene

function at 48-96 hours after injection. Tumor cells are cultured in G418 or FACS sorted (for GFP expression) to enrich for cells that express the phage transducing gene. The ligand encoding sequences are amplified from selected cells using PCR as described for *in-vitro* screening. As in *in-vitro* screening, repeated rounds of infection
5 and rescreeing are performed at increasing screening stringency to obtain the most efficient gene delivery ligands.

From the foregoing it will be appreciated that, although specific
embodiments of the invention have been described herein for purposes of illustration,
10 various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

CLAIMS

We claim:

1. A method of identifying in a library of bacteriophages expressing heterologous peptides or proteins a bacteriophage that binds to a cell surface receptor and internalizes, comprising:
 - (a) contacting a library of bacteriophages expressing a plurality of peptides with a cell, wherein the bacteriophage genome carries a gene encoding a detectable product; and
 - (b) detecting the product;
thereby identifying a bacteriophage expressing a heterologous peptide that binds to a cell surface receptor and internalizes.
2. A method of isolating cells that have internalized a bacteriophage present in a library of bacteriophages expressing heterologous peptides or proteins, comprising:
 - (a) contacting a library of bacteriophages expressing a plurality of peptides with a cell, wherein the bacteriophage genome carries a gene encoding a detectable product; and
 - (b) detecting the product;
 - (c) isolating cells that express the product.
3. A method of selecting a bacteriophage expressing a heterologous peptide that binds to a cell surface receptor and internalizes, comprising:
 - (a) contacting a library of bacteriophages expressing a plurality of peptides with a cell, wherein the bacteriophage genome carries a gene encoding a detectable product;
 - (b) detecting the product; and
 - (c) recovering the bacteriophage gene encoding the peptide from cells expressing the product;
thereby selecting a bacteriophage expressing a heterologous peptide that binds to a cell surface receptor and internalizes.
4. A method of selecting a bacteriophage expressing a heterologous peptide that binds to a cell surface receptor and internalizes, comprising:
 - (a) contacting a library of bacteriophages expressing a plurality of peptides with cells, wherein the bacteriophage genome carries a gene encoding a selectable product;
 - (b) incubating the cells under selective conditions; and

(c) recovering the bacteriophage gene encoding the peptide from the selected cells;

thereby selecting a bacteriophage expressing a heterologous peptide that binds to a cell surface receptor and internalizes.

5. The method according to any one of claims 1-4, wherein the library is a cDNA library.

6. The method according to any one of claims 1-4, wherein the library is an antibody gene library.

7. The method according to any one of claims 1-4, wherein the library is a random peptide gene library.

8. The method according to any one of claims 1-4, wherein the detectable product is selected from the group consisting of green fluorescent protein, β -galactosidase, membrane bound protein, secreted alkaline phosphatase, chloramphenicol acetyltransferase, luciferase, human growth hormone and neomycin phosphotransferase.

9. The method according to any one of claims 1-4, wherein the cell surface receptor is FGF-R or erbB2.

10. The method according to any one of claims 1-4, wherein the cells are tumor cells or endothelial cells.

11. The method according to claim 1, wherein the method further comprises isolating cells expressing detectable product.

12. The method according to any one of claims 2-4, wherein the cells are isolated by flow cytometry.

13. The method according to any one of claims 1-4, wherein the bacteriophage are filamentous phage.

14. The method according to any one of claims 1-4, wherein the bacteriophage are lambdoid phage.

METHODS USING PHAGE DISPLAY FOR SELECTING INTERNALIZING LIGANDS
FOR GENE DELIVERY

ABSTRACT OF THE DISCLOSURE

A bacteriophage system is presented for selecting internalizing ligands for gene delivery. The bacteriophage carries a reporter or selectable marker and presents a ligand on its surface.

C:\PRIZM\429-430\430P1-ap.doc\F